

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Miri Seiberg et al.

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Examiner: Yong S. Chong

For : SOY DEPIGMENTING AND SKIN CARE COMPOSITIONS

DECLARATION OF YAPING HU, PH.D.

I, Yaping Hu, am a Principal Scientist in the Skin Research Center at Johnson & Johnson Consumer Companies, Inc. My education includes a Ph.D. in biology from Rutgers University, NJ, and a B. S. in biology from Jimei University, Xiamen, China. My curriculum vita is attached hereto as Exhibit 1.

I hereby declare:

1. This Declaration is respectfully submitted to describe the trypsin inhibitory activity of a soy composition made in accordance with the above-captioned patent application and in accordance with the publication of "Kelly" (WO 99/36050).
2. In accordance with the data set forth in Table 1 and paragraphs 5 below, the soybean composition made in accordance with Kelly exhibited no trypsin inhibitory activity. The soybean composition made in accordance with the above captioned patent application, at 0.2% concentration, exhibited trypsin inhibitory activity of about 32.58% inhibition. This conclusion is based upon the experiment set forth in sections 3-5 below.
3. In order to create soy compositions in accordance with the Kelly publication, I purchased premium dried soybeans (Hua-Mei Brand) from the Great Wall oriental food supermarket in Franklin Park, NJ, and made the following soybean preparations:
 - A. Soybean was ground for 1 minute using a Warring blender (Dynamics Corporation of America, New Harford, CT 06057). Soybean powder (1g) was soaked overnight at room temperature in 50 ml of deionized water (Sample A, 2% soy)

B. Soybean was ground for 1 minute using a Warring blender (Dynamics Corporation of America, New Hartford, CT 06057). Soybean powder (1g) was soaked overnight at room temperature in 50 ml of 60% ethanol (Sample B, 2% soy). Soybean extract (4 ml, sample B) was then heated on a hot plate (Corning PC-620D, Corning Incorporated life Sciences, Acton, MA 01720) for 1 hour to remove ethanol, and then reconstitute in deionized water to 4 ml.

4. I diluted the Soybean extracts (Samples A and B, each contains 2% soy) 10 times in 1x Phosphate buffered saline (MarTek Corporation, Ashland, MA) to 0.2% soy and I measured the inhibition of trypsin-induced cleavage of a fluorescent casein peptide using the EnzChek™ protease assay kit, following the manufacturer's instructions (EnzChek™ Protease Assay Kits Product Information, Molecular Probes, Eugene OR). Soy preparations were incubated with 100 units of trypsin (Sigma, St. Louis, MO) dissolved in digestion buffer provided in the assay kit. A pure serine protease inhibitor (soybean trypsin inhibitor, from Sigma, St. Louis, MO) was used as a positive control. BODIPY FL casein stock solution (1.0 mg/ml) was prepared by adding 0.2 mL of deionized water to the vials supplied with this substrate (provided in kit), then made a final working solution by combining with 19.8 ml of 1x digestion buffer. Following incubation of the trypsin, with or without the test material, with the BODIPY fluorescent casein substrate at room temperature for one hour, fluorescence was measured (excitation 485 nm /emission 530 nm on a SpectraMax® Gemini microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA) using Softmax® Pro 3.0 software (Molecular Devices Corporation).

5. Table 1 shows that soy preparation prepared according to the above captioned patent application, and pure STI from Sigma, exhibit trypsin inhibition activity. In contrast, sample B, made according to Kelly, does not have trypsin inhibition activity.

Table 1

Samples	Trypsin inhibitory activity (%)	SD
STI .05%	91.45	0.60
STI .01%	56.47	5.47
STI .005%	20.39	1.66
Sample A, 0.2% soy	32.58	1.67
Sample B, 0.2% soy	1.44	2.14

6. Flavonoid compounds are present in soybeans, which are found in nature, in very tiny amounts. For example, the level of total flavonoids in soybeans as found in nature has been reported as approximately 6.81 mg/g, which is 0.68% on a weight/weight basis (Hanan A.A. Taie, El-Mergawi and Radwan, Isoflavonoids, Flavonoids, Phenolic Acids Profiles and Antioxidant Activity of Soybean Seeds as Affected by Organic and Bioorganic Fertilization, American-Eurasian J. Agric. & Environ. Sci., 4 (2): 207-213, 2008, hereinafter “Hanan et al.”). Isoflavones are present in soybeans in nature in the amount of from about 1.261 mg/g (0.13%) to about 3.886 mg/g (0.39%), (Liu, Keshun, *Soybeans, Chemistry, Technology and Utilization*, Aspen Publishers, Inc. Gaithersburg, Maryland, 1999, pp. 83-92, hereinafter “Liu”). Quercetin, the most abundant flavonol, is present in soybeans in nature in the amount of about 0.152 mg/ml (0.015%) (Hanan, et al.). Flavonones are not reported to be found in soybeans. (Hanan, et al.; Liu; Messina, Mark J., Legumes and soybeans: overview of their nutritional profiles and health effects, *Am J Clin Nutr* 1999; 70 (suppl):439S–50S). Copies of the aforementioned references are attached hereto as Exhibit 2.

7. Non-denatured soy compositions made in accordance with the above-captioned patent application were analyzed at my direction. These compositions contain genestin and daidzin, the two major isoflavones in soy, in the amount of about 2.14 mg/g (0.21%) to about 2.26

mg/ml (0.23%) in total, glycitin at about 0.06 mg/ml (0.006%), and daidzein, glycitein and genistein at levels lower than 0.01 mg/ml (0.001%).

8. Tokuyama (JP 5-320061) teaches the use of legume extracts as "an active oxygen elimination agent that is safe and inexpensive and that can be used in a broad range of fields such as medicinal drugs, food products and cosmetic products". Tokuyama teaches of two groups of extracts: A) organic extractions of legumes, which concentrate the isoflavones. Such organic concentrations are commercially available (e.g. from Sigma, see paragraph 10 below); and B) aqueous extracts of legumes, which are boiled (see paragraph 0012 in Tokuyama) and therefore are denatured.

9. I measured the soybean trypsin inhibitory activity of two commercial isoflavone preparations, genestin and of daidzin. These preparations are commercially available and are made in accordance with Tokuyama. Genestin and daidzin, were tested at concentrations equal or greater than five times the concentrations of these two isoflavones in the non-denatured soybean compositions made in accordance with the above-captioned patent application. These isoflavones, at levels that are five times higher than their concentrations in the above-captioned patent application, did not exhibit trypsin inhibitory activity in any measurable amount. This conclusion is based upon the experiment set forth in paragraph 10-12 below.

10. Soy isoflavones, genistin and daidzin, were purchased from Sigma Aldrich (St Louis, MO). A soy suspension (2%) was prepared in PBS using Devansoy high sucrose powder of the whole soybean (Dupont, DE). Non-denatured soy powder was dispersed in PBS, and was sonicated on ice, by an ultra-sonic horn, for 3 times, 10 seconds each (Sonics, Vibracell VC500, Sonics & Materials Inc, Newton, CT). The stock suspension was further diluted in PBS as needed.

11. HPLC analyses of isoflavone contents in a non-denatured soymilk powder and in a non-denatured soy extract (2%) were performed twice at my direction. Six major soy isoflavones

(daidzin, glycitin, genistin, daidzein, glycitein, and genistein) were quantified using a modified method from Klump *et al* (Klump et al., Determination of isoflavones in soy and selected foods containing soy by extraction, saponification, and liquid chromatography: collaborative study. J.AOAC Int. 2001, 84, 1865-1883). Of the six isoflavones, only daidzin, glycitin, and genistin were detected in the soy powder, with the amount of glycitin at only approximately 5% of either daidzin or genistin. In the 2% soy extract, only daidzin and genistin were detectable. It was determined that 0.2% of the non-denatured soy extract contains 1.5 µg/ml of genistin (1x) and 1.2 µg/ml of daidzin (1x).

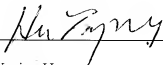
12. For the studies described below, I used the amounts of genistin and of daidzin that are found in the 0.2% non-denatured soybean extracts, and defined these amounts (1.5 µg/ml of genistin and 1.2 µg/ml of daidzin) as 1X. I also used genistin and daidzin at 5 times higher concentrations than those found in the 0.2 % non-denatured soybean extracts, and defined these amounts (7.5µg/ml of genistin and 6.0µg/ml of daidzin) as 5X. The effect of these 1X and 5X genistin and daidzin concentrations was compared with the effect of 0.2% non-denatured soybean extracts (which contains 1X of genistin and daidzin). Trypsin inhibitory activity was measured as described in paragraph 4 above.

13. Table 2 shows that soy preparation prepared according to the above captioned patent application, and pure STI from Sigma, exhibit trypsin inhibition activity. In contrast, genistin and daidzin, two major soy isoflavones, do not have trypsin inhibition activity.

Table 2

Samples	% Inhibition of Trypsin
STI at 0.05%	100%
STI at 0.005%	20.18%
Soy at 0.2%	17.60%
Genistin at 1.5 µg/ml (1X)	-6.91%
Genistin at 7.5 µg/ml (5X)	-7.79%
Daidzin at 1.2 µg/ml (1X)	-6.30%
Daidzin at 6 µg/ml (5X)	-6.14%

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Dr. Yaping Hu

12-17-2010

Date

Exhibit 1

CURRICULUM VITAE

Ya-Ping Hu

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Education:

Ph.D., Biological Science, Rutgers University. 1992.

M.S., Biological Science, Chinese Academia of Sciences. 1982.

B.S., Biology, Jimei University, Xia Men, China. 1976.

Professional Experience:

06/2007- recent, Principal Scientist, Johnson and Johnson, Skin Research Center
Skillman, NJ 08558.

- Development of in vitro skin explants model for skin research.
- Skin pigmentation.
- Skin aging.

04/1999-05/2007, Research and Teaching Specialist II, Center for Advanced
Biotechnology and Medicine, UMDNJ, Piscataway, NJ.

- Create mutant mouse model for study of gene functions.
- Embryonic stem cell (ESC) culture, maintaining various mutant ESC strains and stocks;
- Training the grad-students and post-docts in molecular cloning, gene targeting strategy and ESC culture techniques.

05/1997-04/1999, Research Associate, Dept. of Surgery, UMDNJ, Piscataway, NJ.

- Using *in vitro* model to study the cell-matrix and cell-material interaction.
- Application of molecular biology techniques to characterize the platelet alpha-actinin. Generated GFP and His-tagged alpha-actinin fusion proteins, point and truncation mutants, expression of fusion protein in bacteria and mammalian cells.

05/1995-05/1997, Postdoctoral Fellow, Center for Human and Molecular Genetics, UMDNJ, Newark, NJ.

- Positional cloning, mapping and linkage analysis of human non syndrome deafness. Research efforts involve handling of blood samples collected from several families and genotyping with chromosome markers.

08/1992-05/1995, Postdoctoral Researcher, Molecular Evolution Program, Louisiana State University, Baton Rouge, L.A. (8/92-5/95; Dr. David Foltz)

- Developed single copy DNA markers for species and population identification.
- Investigation of population genetics in early life stages and geographic populations of marine organisms

01/1987-06/1992, Graduate Student, Center for Theoretical and Applied Genetics, Rutgers University, New Brunswick, NJ),.

- Application of molecular biology techniques to species identification and genetics analysis.
- Genetics study including *in vitro* fertilization, embryo culture, polyploid induction and karyotyping.

Publications:

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Context-dependent neuronal differentiation and germ layer induction of Smad4^{-/-} and Cripto^{-/-} embryonic stem cells. Sonntag KC, Simantov R, Bjorklund L, Cooper O, Pruszk J, Kowalke F, Gilmartin J, Ding J, Hu YP, Shen MM, Isacson O. Mol Cell Neurosci. Mar;28(3):417-29, 2005

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A point mutation in the CytB gene of mtDNA associated with complex III deficiency in ischemic cardiomyopathy. Marin-Garcia, J., Y.P. Hu, R. Ananthakrishnan, M.E. Pierpont, G.L. pierpont and M. J. Goldenthal. Biochemistry and Molecular Biology International, 40(3): 487-495, 1996.

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1996.

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Vrijenhoek and R.A. Lutz. J. Heredity, 84: 254-258, 1993.

Electrophoresis and genetic analysis of larval bivalve mollusks. Hu, Y.P., R.A. Lutz
and R.C. Vrijenhoek. Mar. Biol. 113: 227-230, 1992.